REMARKS

Claims 1 and 10-11 have been amended. Antecedent support in the specification for the amendment to the first paragraph of claim 1 is found in the paragraph beginning on page 3, line 42 and ending on page 4, line 5. Antecedent support in the specification for the amendment to subsection d) of claim 1 is found on page 7, lines 7 to 15, and in particular at lines 10 to 13.

The examiner has objected to claim 10 under 37 CFR 1.75(c) for failing to further limit the subject matter of the previous claim from which it depends. Applicants respectfully submit that changes in enzymatic activity other than regioselective, chemoselective and stereoselective changes are possible. Such changes include, but are not limited to, higher activity and better binding to a substrate or to different substrates having a particular chemical group. Favorable reconsideration of this rejection is solicited.

The examiner has rejected claims 1-2 and 5-7 under 35 USC 102(b) as anticipated by Greener et al. Claim 1 has been amended to more clearly delineate the present invention. Applicants respectfully request the examiner to reconsider this rejection in light of the above amendments and in light of the following remarks.

Applicants respectfully submit that the rejection maintained by the examiner results from a mutual misunderstanding concerning the phrase "alteration of substrate specificity." As indicated in the paragraph beginning on page 3, line 42 and ending on

page 4, line 5 of the specification, alteration of substrate specificity for purposes of the invention as claimed is defined as the development of an affinity in an enzyme that did not exist before. Such a new affinity, or "new substrate specificity," as the examiner has referred to such a newly developed ability, enables the enzyme to convert the substrate with which it has this new affinity.

The cited paragraph of the present specification states this in the following way. "[T]he [resulting] enzymes . . . are able to convert substrates which they were previously unable to convert." The disclosure states this in terms of k_{cat} and k_M, in this way. The affinity for these new substances, i.e., the ratio of k_{cat} and k_M, "is zero or almost zero," meaning that the enzyme possesses no or very little affinity for the particular substrate. There is essentially no substrate specificity for that substrate and, therefore, as stated in the specification, "catalysis does not occur." After the process of the present invention, the ratio of k_{cat} and k_M becomes greater than zero for that particular substrate, and the enzyme is then "able to convert" the substrate which it was "previously unable to convert."

The examiner's rejection of claims 1-2 and 5-7 as anticipated under 35 USC §102(b) has been based on the examiner's assumption that "alteration in specific activity" for the purposes of the present invention is other than that given in the language of the present specification. The examiner refers to the "commonly held definition of substrate specificity in the art" (office action of December 11, 2000, page 6,

and office action of July 12, 2000, page 8) in the §102(b) rejection over Greener, and does not acknowledge or comment on the specific definition utilized and set forward by applicants in the present specification. Greener screens enzyme products for changes in specificity for a particular substrate, for example increased specificity giving higher rates of catalysis, or decreased specificity giving lower rates of catalysis. Greener screens the enzyme products for affinities and specificities inherent in the enzymes *prior* to the mutagenesis steps. Greener does not screen the enzyme products for the ability to convert substrates they were previously unable to convert, and therefore Greener does not teach the process of the present invention as claimed. Had the examiner acknowledged the semantic difference employed and taught by applicants in their specification, the distinction between Greener and the present invention would have become apparent more quickly.

The present invention as claimed employs a method for inducing substrate specificity changes in enzymes such that the enzymes attain a completely new substrate specificity, one not previously held prior to performance of the claimed method. The present invention as claimed also screens for and isolates microorganisms that express these enzymes with new substrate specificities, utilizing the new substrate as an indicator molecule. The steps of this method, and the end product attained, are significantly different from the steps of Greener and the end products attained in that disclosure.

Greener employs a method for inducing substrate specificity changes in enzymes such that the enzymes have a changed specificity, i.e., increased or decreased specificity, or complete loss of specificity, for a substrate on which they were previously able to act. Greener also screens for and isolates microorganisms that express these enzymes with changed specificity for the same substrate, utilizing the substrate upon which the enzymes were previously able to act. Greener uses a different screening step, uses different substrates to screen for the desired enzyme products, and ultimately isolates different microorganisms than the present invention as claimed.

In the process of the present invention as claimed, genes coding for a specific enzyme that have been mutated utilizing the microorganism *Escherichia coli* XL1 Red are isolated and introduced into new hosts. These hosts are not mutator strains and must not contain the ability to convert the new substrate for which new substrate specificity is desired. The host microorganisms are screened on a selection medium that contains a substrate that the unmutated enzyme cannot convert. Those host microorganisms that contain a mutated enzyme that *is* able to convert this new substrate are isolated so that the mutated enzyme may be employed to convert the new substrate.

In the example of the present specification, the gene for an esterase originally derived from *Pseudomonas fluorescens* (PFE-wt) and inserted into a plasmid (plasmid

2792.1) was mutated in *E. coli* XL1 Red. Prior to the mutagenesis step, the esterase enzyme was demonstrated to have an inherent ability to convert ethyl acetate to acetic acid. The unmutated esterase was also demonstrated *not* to have the ability to convert certain 3-hydroxy esters at all. The transformed *E. coli* XL1 Red microorganisms were incubated, thereby introducing mutations into the PFE-wt gene, and some of the resulting mutated genes (PFE-U3, for example) were re-inserted into new *E. coli* XL1 Red microorganisms for a second mutagenesis round to increase the amount of mutation introduced. The mutated genes were then isolated and inserted into *E. coli* DH5α microorganisms, which have no ability to convert the 3-hydroxy esters noted above.

These *E. coli* DH5α microorganisms were screened on two different selection media. One, "a)", included indicators and the 3-hydroxy ester substrate II-1 shown on page 9, lines 43-45 of the specification. The other, "b)", included indicators and the 3-hydroxy ester substrate II-3, also shown on page 9, lines 43-45 of the specification. *E. coli* DH5α microorganisms with esterases possessing new substrate specificities for these two 3-hydroxy ester substrates (i.e., "positive clones") were identified, and certain clones were isolated and induced to produce the mutated esterase. Clone PFE-U3 esterase was then demonstrated to stereoselectively convert the 3-hydroxy ester substrate II-3, and at this point the PFE-wt esterase was demonstrated to be unable to convert this new substrate.

This process of the present invention screens microorganisms containing mutated enzyme genes for the ability to convert substrates which the enzymes were unable to convert prior to the mutagenesis step. The selection medium used for the screening step contains the new substrate and may contain other indicator compounds to demonstrate the ability of the mutated enzymes to convert the new substrate. Those microorganisms into which the genes for enzymes with the new substrate specificity have been inserted are then isolated and the enzymes are utilized to convert the new substrate. Greener does not teach this process.

In the process disclosed by Greener, genes coding for a specific enzyme that have been mutated utilizing the microorganism *Escherichia coli* XL1 Red are also isolated and introduced into new non-mutator host bacteria. The host bacteria are screened on a selection medium that contains a substrate that the unmutated enzyme has been demonstrated to have the ability to convert. Those host bacteria that contain a mutated enzyme that shows some *change* in the ability to convert this previously demonstrated substrate are then isolated. Applicants argue that the method of the present invention as claimed is patentably distinct from this disclosure, as Greener does not teach the elements of screening for new specificities, using substrates for which the enzymes have never had demonstrable specificity, and isolating the microorganisms containing mutated genes for enzymes with the new substrate specificity.

Greener teaches a method whereby mutant enzymes (and regulatory proteins) are detected by an increase, decrease or loss of a previously demonstrated specificity. The only example in Greener that is close to the present invention as claimed, in that it screens directly for enzyme specificity and activity as opposed to screening for the growth of the transformed host colonies, is found in paragraph 8 on page 383. In that example, an alkaline phosphatase gene is isolated from the hyperthermophilic archebacterium *P. furiosus*, and introduced into *E. coli* strain HB3, which lacks an alkaline phosphatase gene. In this host, and at the particular temperature of 37°C, the alkaline phosphatase produced has a very low activity for conversion of BCIP, an indicator substrate. Greener indicates that alkaline phosphatase of *E. coli* is able to convert this substance at this particular temperature.

Greener implicitly expresses that the lower activity of the *P. furiosus* alkaline phosphatase is due to the lower temperature employed, and that the alkaline phosphatase would be better able to convert this substance at the high temperatures at which the phosphatase normally acts (see the phrase "very low activity *at* 37°C", paragraph 8, page 383, emphasis supplied, and also the language "having higher specific activity at 37°C *or higher specific activity at all temperatures*", paragraph 8, page 384, emphasis supplied). The method of Greener does not seek to change the substrate specificity of this enzyme, rather it seeks to increase the range of temperatures under which this enzyme can act on this substrate for which it already

possesses specificity.

Greener transformed *E. coli* XL1 Red bacteria with a plasmid containing the *P. furiosus* alkaline phosphatase gene and incubated these to induce mutation in the gene. The plasmids were isolated and introduced into the *E. coli* HB3 hosts, which were screened in selection medium containing the BCIP indicator substrate. Microorganisms with mutated enzymes having an increase in specificity at 37°C were isolated and the plasmids were removed and run through another mutation cycle. The resulting mutated genes were isolated and introduced again into *E. coli* HB3 hosts, which were again screened on a selection medium containing the BCIP indicator substrate. Again, those microorganisms demonstrating an increase in specificity for the BCIP indicator substrate at the temperature of 37°C were isolated.

From this example, applicants submit that Greener does not teach the elements of the present invention as claimed as they have been outlined in the previous pages. Greener isolates a gene that produces an enzyme that is able to break down a certain indicator substance at an elevated temperature. Greener then mutates this gene using a bacterial mutator host and transforms the mutated genes into bacterial hosts lacking the particular enzymatic activity previously held by the enzyme. Greener incubates these bacterial hosts on a selection medium containing the indicator substance that the gene product previously had affinity for, and isolates those hosts with enzymes that show an increased specificity.

The present invention as claimed isolates an enzyme gene, mutates it, and screens the transformed non-mutating hosts for enzyme activity not previously held by that particular enzyme. The present invention as claimed differs from Greener in the substrate specificity to be created relative to that specificity held by the enzyme prior to mutagenesis. The present invention as claimed also differs from Greener in the screening method undertaken, and the composition of the selection medium utilized in this screening method step to identify enzyme mutations. The present invention further differs from Greener in the ultimate products isolated from the mutagenesis and screening process steps, in the products' demonstrated and desired substrate specificities. As Greener does not teach all of the elements of the present invention, applicants respectfully assert that the cited reference cannot anticipate the present invention as claimed under 35 USC §102(b) (see *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987)).

The examiner has rejected claims 4 and 10 - 11 under 35 USC §103(a) as obvious over Greener in view of Wilks et al. Applicants respectfully request the examiner to reconsider this rejection in light of the following discussion.

Applicants assert that Wilks, when combined with Greener, does not render the present invention as claimed obvious under 35 USC §103(a). The examiner has the burden of setting forward a *prima facie* case of obviousness, and applicants submit that this burden has not been met in the instant rejection. Applicants submit that the

reasoning set forward by the examiner has not established that one skilled in the art would be motivated by either of the Greener or Wilks disclosures alone, or the two references together to attempt the process of the present invention as claimed. Applicants submit that sufficient expectation of success has not been demonstrated based on those disclosures and scientific principles generally known by those skilled in the art.

As indicated above, Greener discloses a process whereby mutations in a gene having specificity for a particular substrate are introduced, and the resulting gene products are screened for an increase in that specificity. The difference in specificity is confined to specificity for a single substrate, and applicants submit that such a change in specificity would appear to one skilled in the art to be relatively slight, as the basic specificity is kept and only slightly modified.

Random mutagenesis by its very nature introduces more disorder into a system, and the further removed a particular structure is from that structure in place prior to the mutagenesis, the less likely that structure is to be created by this process. Using this application of the law of entropy, the likelihood of creating an entirely new substrate specificity in an enzyme through random mutagenesis would be far less than increasing the specificity for a particular substrate. In the latter case, much of the necessary structure is already possessed by the enzyme, and any changes introduced are much more likely to slightly alter this structure so as to enhance specificity than to make the

larger alterations needed to create an entirely new specificity. The randomness and chaotic nature of mutagenesis argue against utilization of such a method to create a new structure in an existing enzyme. One skilled in the art would find an increase in prior specificity surprising enough, and might think it obvious to *try* to create a new specificity in an enzyme, but would not have a sufficient expectation of success to be motivated to undertake this process.

Without some specific suggestion, either in Greener or in the knowledge generally available to one skilled in the art, applicants submit that such a person would not see random mutagenesis as a potentially successful method of introducing a new substrate specificity into an enzyme. Applicants can find no such suggestion in Greener, and as discussed above, find that general scientific knowledge held by molecular biologists teaches away from the use of random mutagenesis to produce new substrate specificities. Therefore, applicants submit that the disclosure in Greener would not render the present invention as claimed obvious under 35 USC §103(a) were it to be applied in isolation .

Applicants further respectfully assert that Wilks also fails to suggest to one skilled in the art motivation to create the present invention as claimed. Wilks discloses a process of rational protein design in which a computer-generated, three-dimensional model of an enzyme/new substrate complex, including necessary cofactors, is created and analyzed, and where the protein is then modified through site-specific

mutagenesis. Applicants assert that this does not suggest the desirability of random mutagenesis, where changes in nucleic acid and amino acid structure are introduced randomly, without design, into a gene and its corresponding enzyme to create a new enzyme specificity.

Initially in these remarks, applicants would like to bring to the examiner's attention an apparent oversight. In the examiner's office action dated March 23, 2000, the examiner cites language from Wilks, characterizing that reference as "equat[ing] 'random mutagenesis and screening' with 'rational design and construction'." The examiner directs applicants to page 561, first paragraph, of Wilks for this language. Applicants have been supplied by the examiner with a copy of the Wilks reference that begins with a page 150 and ends with a page 154, and there accordingly being no page 561 found therein. Applicants further have not found either of the phrases "random mutagenesis and screening" or "rational design and construction" anywhere in the supplied reference. Applicants request the examiner to more particularly point out the language relied on to create the quotation taken from the above-mentioned office action. If the examiner could either correct the page number to reflect the Wilks reference that has been supplied, and specifically indicate where on that page the language appears, or provide a copy of the Wilks reference that includes page 561, applicants would willingly respond with greater precision to the argument set forward by the examiner.

To the extent that they are able, applicants offer the following discussion of the examiner's argument contained in the quotation cited above. In the introduction of the Wilks reference actually in possession of the applicants, vague allusion is made to random mutagenesis in the statement that "[n]atural enzymes are under evolutionary pressure to transform compounds found in the same cell as the enzyme." Evolutionary pressure is commonly counteracted through capitalization on random genetic mutation, and therefore Wilks does, indeed refer to random mutation in the introduction. However, in opposition to the examiner's statement that Wilks *equates* random mutagenesis with rational enzyme redesign, this language indicates a stabilizing effect exerted on an enzyme to meet the demands of its immediate environment, rather than a diversifying effect of random mutation to produce an enzyme a with novel substrate specificity.

Wilks underscores the relative uselessness of such random mutation for the purposes of enzyme redesign by stating that "only fortuitously [will these enzymes] be found useful for the chemoenzymic conversion of 'unnatural' intermediates required for chiral pharmaceuticals synthesis" (introduction, page 150 of the Wilks reference supplied to the applicants). In other words, evolutionary pressure, and the random mutagenesis that drives it, will not, except "only fortuitously," produce enzymes with new substrate specificities of the type envisioned and taught by Wilks. Applicants submit that Wilks is in essence stating that rational drug design is the only method (at

least when compared to random mutagenesis) for creating enzymes with new substrate specificities as desired. This point is itself underscored by Wilks' statement that the extant "pessimistic view" of enzyme redesign (even when random mutagenesis was a known alternative) was only "countered by a . . . switch in substrate specificity . . . which transformed a lactate dehydrogenase . . . into a malate dehydrogenase" achieved by Wilks "rationally" (page 150, first paragraph under "Enzyme Redesign").

Rational protein redesign as disclosed by Wilks requires a rigorous and complete understanding of the structural detail of the enzyme to be redesigned. Illustrative of this requirement is the statement in the disclosure that "although [Wilks et al.] have a raw resolution x-ray structure of the D-LDH from a thermotolerant *Lactobacillus* . . . the structural detail is not yet sufficient for rational redesign" (p. 151). The requirement for structural knowledge, and the precision engineering that computer modeling allows, gives Wilks the ability to quickly and accurately make perceived, optimal alterations in an enzyme structure. Applicants submit that one skilled in the art would likely be motivated by this disclosure to develop an improved library of structural information on enzymes of interest for protein redesign. Lack of control over the structural changes actually produced, and the time intensive and potentially profitless nature of the random mutagenesis method would most likely discourage one so skilled to employ that process for enzyme redesign.

Wilks sets out to design a protein that will accept specific variations of a 2-ketoacid structure. To do this in such a manner that the enzyme will act on these new substrates with the highest certainty, Wilks generates a three dimensional computer model of the enzyme in its folded, active state. Into this enzyme model Wilks places the enzyme's cofactors, and the desired new substrate. Wilks then analyzes the structure, uses "chemical intuition," and manipulates the model's amino acid structure using software to determine a potentially successful alteration. Then the appropriate, precise, changes are made in the nucleic acid of the corresponding gene to reflect the deduced changes that will give the highest potential success. This process enables an individual with a great amount of data to alter the amino acid structure precisely so as to create a new specificity with a very high possibility of success. Applicants submit that such a disclosure would motivate one skilled in the art away from random mutagenesis, a process with no control over precise changes in amino acid structure and only a very low expectation of success.

Without some specific statement to the contrary in the disclosure, applicants assert that the process of Wilks would not suggest to one skilled in the art that changing an enzyme's substrate specificity by random mutagenesis has any real probability of success. The entropic degradation of structure, the imprecision and the time consumption related to a random mutagenesis method for enzyme redesign all run counter to the precision, accuracy and quickness of Wilks' rational redesign method.

Applicants respectfully submit that the examiner has set forward no knowledge generally held by one skilled in the art that would be sufficient to link the disclosure of Wilks with the random mutagenesis process of the present invention. Applicants assert that no suggestion, either implicit or explicit, from either Wilks or from the knowledge generally held by one skilled in the art, has been set forward to give a reasonable expectation of success for creating a new substrate specificity in an enzyme through random mutagenesis. Accordingly, applicants submit that the present invention as claimed cannot be obvious under 35 USC §103(a) over Greener in view of Wilks, or over Wilks in isolation.

In view of the foregoing amendments and remarks, applicants consider that the rejections of record have been obviated and respectfully solicit passage of the application to issue.

Please charge any shortage in fees due in connection with the filing of this paper, including Extension of Time fees to Deposit Account No. 11-0345. Please credit any excess fees to such deposit account.

Respectfully submitted,
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Claims as of 3/12/01 in BORNSCHEUER et al., SN 09/161,680, OZ 0050/48429/UP

1. A method for altering the substrate specificity of an enzyme wherein the affinity for a new substrate, measured as k_{cat} / k_M, rises from zero or almost zero, to greater than zero, such that the enzyme converts the new substrate, which method comprises the steps of:

a) introducing a DNA which comprises a copy of the gene coding for the enzyme into the Escherichia coli strain XL1 Red or into a functional derivative,

b) incubating the transformed Escherichia coli strain XL1 Red or its functional derivative to generate mutations in the enzyme gene,

c) transferring the mutated DNA from the strain XL1 Red or its functional derivative to a microorganism which has no impeding enzyme activity,

d) incubating this microorganism to detect the enzyme activity on or in at least one selection medium which comprises at least one enzyme substrate to recognize altered substrate specificity of the enzyme, with or without other indicator substances,

e) selecting the microorganisms which show an alteration in the substrate specificity, said microorganisms in steps b), d) and e) being a member selected from the group consisting of bacteria, fungi and yeasts.

2. A method as claimed in claim 1, wherein steps (a) to (e) are performed several times in sequence by reisolating and retransforming the DNA From the microorganisms selected in step (e) to the strain *Escherichia coli* XL-1 Red or its functional derivative.

4. A method as claimed in claim 1, wherein the bacteria are Gram-positive or Gram-negative bacteria.

5. A method as claimed in claim 1, wherein a hydrolase is used as enzyme.

6. A method as claimed in claim 1, wherein an enzyme selected from the group consisting of proteases, lipases, phospholipases, esterases, phosphatases, amidases, nitrilases, ether hydrolases, peroxidases and glycosidases is used.

7. A method as claimed in claim 1, wherein a lipase, esterase or nitrilase is used.

8. A method as claimed in claim 1, wherein the alteration in the substrate

specificity results in a selective enzymatic activity.

9. A method as claimed in claim 8, wherein the alteration in the substrate specificity results in regio-chemo- or stereoselective or regio-chemo and/or stereoselective enzymatic activity.

10. A method as claimed in claim 1, wherein the alteration in the substrate specificity is selected from the group consisting of a regioselective, chemoselective or stereoselective alteration.

11. A method as claimed in claim 10, wherein the alteration in the substrate specificity results in a stereoselective enzyme activity.